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Protective antigens of *Mycobacterium habana* are distributed between peripheral and integral compartments of plasma membrane: a study in experimental tuberculosis of mouse

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Abstract

Mycobacterium habana, a cultivable nonpathogenic mycobacterium provides appreciable resistance in mouse against *M. tuberculosis* infection. This study is aimed at identification and characterization of protective antigens of *M. habana*. Protective potential of antigens of cell wall (CW), cell membrane (CM), cytosol (CS) and peripheral and integral compartments of the membrane fraction of *M. habana* was explored against experimental tuberculosis in mouse. Peripheral and integral membrane proteins were characterized by SDS-PAGE and differential staining with silver and periodic acid. Results reveal that protective antigens are distributed in both peripheral and integral membrane compartments though such effect is dominant in the former. Polysaccharide staining showed that LAM, LM and PIMs have a preference for the detergent phase. Peripheral and integral compartments constitute, respectively, 68 and 31% of the total membrane protein.

Keywords: *Mycobacterium habana*; Protective antigens; Tuberculosis; Mice

1. Introduction

The formidable resurgence of tuberculosis in the wake of AIDS is causing global concern [1,2]. The situation has worsened due to emergence of multidrug resistant strains [3] of *M. tuberculosis*. Controversial status of *M. bovis* BCG [4], the only available vaccine against tuberculosis necessitates search for superior vaccine. Keeping this in view a search for immunoprotective mycobacterial strains was initiated by our laboratory. Among a panel of 19 mycobacterial strains tested to evaluate their protective efficacy in mouse model, three strains namely, *M. habana* TMC 5135, *M. bovis* BCG Phipps TMC 1029 and *M. tuberculosis* $H_{37}Ra$ TMC 201 were found protective. *M. habana* in this and consequent studies has shown better efficacy than BCG [5]. Moreover, *M. habana* also provided protection (in mouse) against different indigenous strains of varying (high to low) virulence procured from South India [6]. Prevalence of such strains was thought to be one of the reasons for the failure of BCG (seed lot 173P2, French strain and seed lot 1331, Danish strain) in clinical trials in South India.

Moreover since whole cell vaccines have their own limitations [7–10], search for independent immunogenic moieties free from the redundant molecules is of greater interest. The response of "Helper" subset of Th lymphocytes (Th cells) which is thought to play a critical role in protection through lymphokines which activate macrophages [11]. Th cell response is focused on a limited number of discrete sites on protein molecules [12]. For this reason, an array of antigenic proteins have been isolated or genetically cloned and characterized from different compartments of mycobacterial cell [13–15]. Therefore, the present study has been carried out as a systematic approach for identifi-

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cation and subcellular localisation of antigens which significantly contribute towards the protective efficacy of *M. habana* against tuberculosis infection.

2. Materials and methods

2.1. Subcellular fractionation of M. habana

Mycobacterium habana, TMC 5135 (maintained on solid L–J medium) was grown in liquid Sauton's medium (containing L-asparagine and glycerol) at 37°C in shaking incubator to obtain log growth in bulk. Defined mid-log phase (10 d) cultures were harvested by centrifugation (4000 × *g* for 20 min, 4°C) and washed three times with sterile normal saline.

Three broadly defined subcellular fractions, viz., the cell wall (CW), cell membrane (CM) and cytosol (CS) were prepared according to the protocol of Brodie [16] by sonication and differential centrifugation, as described elsewhere [17].

2.2. Characterization of the cell membrane fraction

Morphology of the membrane fraction was checked by transmission electron microscopy [18] and purity was assessed by determining the activities of marker enzymes for bacterial plasma membrane, namely, adenosine triphosphatase (ATPase), lactate dehydrogenase (LDH) and glyceraldehyde-3-phosphate dehydrogenase (G-3-PDH) by standard protocols [19,20] in relation to the specific activities of these enzymes in the cytosol.

2.3. Separation of proteins of integral and peripheral compartments of the membrane fraction

Proteins of peripheral compartment of the membrane were separated from those associated with the core of lipid bilayer belonging to integral compartment [21,22] by phase separation using Triton X-114 [11,12]. Briefly, to a suspension of purified membrane pellet, 10 mg protein/ml Tris buffer (10 mM Tris–HCl, pH 7.4 containing 150 mM NaCl and 0.02% NaN_3) 50 µl of precondensed [23] Triton X-114 (in Tris buffer) was added. The solution was mixed and chilled in ice to make a single phase. Residual insoluble material was removed by centrifugation at 2500 × *g* for 10 min at 37°C. The supernatant was kept at 37°C for 30 min. After centrifugation at 3000 × *g* for 10 min at 30°C resultant aqueous and detergent phases were separated. Proteins of both phases were "back washed" (×3), i.e., aqueous phase proteins (APP, 9.5 ml) were treated with 500 µl 20% Triton X-114 and those of triton or detergent phase (DPP, 0.5 ml) with 9.5 ml of Tris–HCl (10 mM Tris, 150 mM NaCl, pH 7.0) to get rid of the inter phase contamination. Proteins in the detergent phase were recovered by precipitation with 3–4 volumes of chilled (−20°C) distilled acetone. Proteins of the aqueous phase were concentrated by lyophilization.

Protein content in various subcellular fractions and subfractions was estimated by modified Lowry's method [24].

2.4. Characterisation of protein profiles

Protein profiles of cytosol, cell wall and cell membrane fractions as well as peripheral (recovered in aqueous phase, APP) and integral (recovered in triton phase, DPP) compartments of membrane fraction were compared by resolving them on 12% acrylamide gels under reducing conditions (SDS-PAGE) [25] and staining with Coommassie blue. For determination of glycosylation, APP and DPP resolved by SDS-PAGE were stained with silver including a periodic acid step [26].

2.5. Comparison of protective efficacy of the antigen preparations.

2.5.1. Immunizations

Four groups of 15 mice (BALB/c) each were immunized intradermally with the following antigens, (1) 6.27×10^8 γ-irradiated *M. habana* cells, (2) fractions derived from equivalent number of bacilli, i.e. cell wall, 20 µg protein/mouse; membrane, 5 µg protein/mouse; and cytosol, 30 µg protein/mouse.

Three groups of 15 mice each were immunized (5 µg protein/mouse) with whole membrane and aqueous and detergent fractions of the membrane.

One group of animals in both the experiments was kept as control (unimmunized) and given 0.1 ml N-saline intradermally.

2.5.2. Assessment of protective immunity to virulent challenge

Three weeks after the immunizations, immunized as well as control mice were challenged intravenously with 10^8 CFUs of *M. tuberculosis* $H_{37}Rv$ (TMC 102). Challenged animals were observed for 30 d for mortality, and mean survival time (MST) was calculated on day 30. Enumeration of bacteria (CFU) in the organs (lung and spleen) of infected animals were done after 60% mortality in the control groups by plating serial ten fold dilutions of organ homogenate on to L–J medium and incubated at 37°C. Colonies were counted after 3 to 4 weeks and were expressed as mean counts from three animals in each group. Colony forming units (per gram of tissue) were converted into Log_{10} values.

Fig. 1. Electron micrograph of negatively stained membrane vesicles (magnification × 82,000). Bar, 100 nm.

2.6. Statistical analysis

Difference in protective efficacy between immunized and unimmunized or between immunized animals was analyzed by one way analysis of variance. p values less than 0.05 ($p > 0.05$) were considered significant.

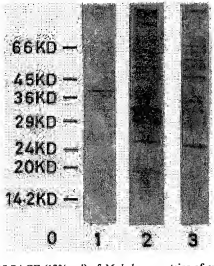

Fig. 2. SDS-PAGE (12% gel) of *M. habana* proteins of cytosol (lane 1, CS), cell wall (lane 2, CW) and cell membrane (lane 3, CM). Molecular weight markers are shown in lane 0.

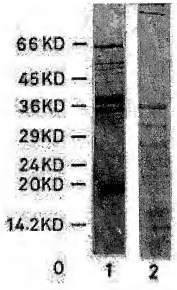

Fig. 3. Triton X-114 partitioning of cell membrane proteins. Separation on 12% acrylamide gel (SDS-PAGE) and staining with Coomassie blue. Lane 0, molecular markers; lane 1, aqueous phase proteins (APP), and lane 2, detergent phase proteins (DPP).

3. Results

3.1. Characterization of the cell membrane fraction

Vesicle structure seen under the transmission electron microscope (Fig. 1) confirmed the membrane morphology. Similarly, specific activities of LDH, ATPase and G-3-PDH found, respectively, 19.75, 10.79 and 4.6 fold higher in the membrane fraction than the corresponding activities in cytosol indicating purity of the membrane fraction.

3.2. Profile of proteins distributed in peripheral and integral compartments of the membrane

Following Triton X-114 extraction, a major proportion (68%) of membrane proteins partitioned in the aqueous phase whereas only about 31% were recovered in the detergent phase. Resolution of CW, CS and CM fractions by SDS-PAGE showed an array of CM proteins (Fig. 2, lane 3) appearing as clear bands at approximately 70, 65, 62, 57, 54, 53, 48, 42, 40, 38, 35/36, 34, 31, 25, 18/19 and 14 kD positions. When compared with the profile of aqueous (APP) and detergent (DPP) phase proteins, it was observed that the 65, 56, 54, 53, 48, 38–40, 35/36, 25 and 18/19 kD proteins partitioned in the aqueous phase (Fig. 3, lane 1) whereas the 38, 34 and 14 kD proteins (and few weakly stained proteins between 70 and 40 kD) were recovered in the detergent phase (Fig. 3, lane 2). Interestingly, in detergent phase some additional pro-

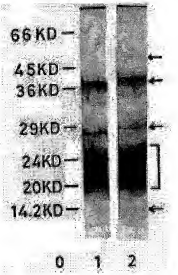

Fig. 4. Separation of DPP on 12% gel (SDS-PAGE) and staining with silver and periodic acid (lanes 1 and 2). Lane 0, molecular markers. Arrows on right hand side indicate major bands.

tein bands were seen which were not visible in the CM fraction; these were 27, 24, 23, 15 and 12/10 kD.

Specific silver staining for polysaccharides (Fig. 4, lane 1 and 2) showed 3 prominent broad bands in the molecular weight range of 38–36, 27 and 25–18 kD in the detergent phase. Two less intense sharp bands were seen at about 47 and 12 kD positions. The broad bands may correspond to the group of lipopolysaccharides, e.g. LAM, LM and PIMs. This staining pattern indicates that lipopolysaccharide conjugated proteins of the membrane have preferentially been recovered in the Triton X-114 phase.

Molecular mass of individual bands was determined by drawing calibration curve with standard molecular markers (Sigma USA).

Table 1
Comparison of protective efficacies of cell wall, cell membrane and cytosolic antigens of *M. habana* against *M. tuberculosis* $H_{37}Rv$ challenge in mouse (results expressed are mean ± S.D. of 3 experiments; values in parentheses indicate fold increase (↑) or decrease (↓) with reference to the control group)

Groups	Viable bacilli count (CFU in Log_{10})		Survival (MST on day 30)
	Lungs	Spleen	
Control	11.690 ± 0.272	8.359 ± 0.349	12.10 ± 1.75
Whole cell	8.329 ± 0.306 (1.40↓)	6.121 ± 0.714 (1.36↓)	24.48 ± 0.97 (2.03↑)
Cell membrane	7.490 ± 0.790 (1.56↓)	5.884 ± 0.525 (1.42↓)	24.76 ± 0.34 (2.04↑)
Cell wall	9.322 ± 0.185 (1.25↓)	8.267 ± 0.372 (1.01↓)	20.13 ± 2.40 (1.66↑)
Cytosol	9.747 ± 0.715 (1.19↓)	8.273 ± 0.890 (1.01↓)	19.20 ± 1.82 (1.58↑)

Table 2
Statistical analysis for significance of variance between unimmunisced and immunized groups (differences, statistically significant $p < 0.05$; highly significant $p < 0.01$ and not significant (NS) $p > 0.05$)

	CFU		MST
	Lung	Spleen	
Control vs whole cell	$p < 0.01$	$p < 0.05$	$p < 0.01$
Control vs cell membrane	$p < 0.01$	$p < 0.05$	$p < 0.01$
Control vs cell wall	$p < 0.01$	NS	$p < 0.05$
Control vs cytosol	$p < 0.05$	NS	$p < 0.01$
Whole cell vs cell membrane	NS	NS	NS
Whole cell vs cell wall	NS	$p < 0.05$	$p < 0.05$
Whole cell vs cytosol	NS	$p < 0.05$	$p < 0.05$
Membrane vs cell wall	$p < 0.05$	$p < 0.01$	$p < 0.05$
Membrane vs cytosol	$p < 0.01$	$p < 0.01$	$p < 0.05$

3.3. Determination of subcellular location of protective antigens

Comparison of protective efficacies of the three subcellular fractions; cell wall, cell membrane and cytosol of *M. habana* against live challenge of *M. tuberculosis* is given in Table 1. Statistical analysis for significance of variance is given in the supplementary table (Table 2). Data indicate that survival was greater and density of viable *M. tuberculosis* bacilli (CFU) in lung and spleen was lower in groups of mice immunized either with whole cell or its fraction(s) as compared to the unimmunized (control) animals. All the differences were statistically significant except for CFUs in the spleen of cell wall and cytosol immunized groups.

In a comparison between mice immunized with whole bacterial cells and those immunized with different subcellular fractions, CM group had the highest survival rate (2.04 times that of the control) which was similar to that of the whole cell group (2.03 times). CFUs in lung and spleen of CM group were found lower than the whole cell group, however these differences were not significant statistically. Survival rates were significantly lower and CFUs in spleen were significantly higher in the CW and CS groups (compared to the whole cell group). Similar findings were observed when data of CW and CS groups were compared with the CM group.

3.4. Localisation of protective protein antigens within the membrane fraction

In the second set of experiments, a comparison of the protective efficacies of membrane proteins partitioned in the aqueous and detergent phases (APP and DPP) was done. Survival and presence of viable bacilli

Table 3
Comparison of protective efficacy of membrane proteins of *M. habana* partitioned in aqueous and detergent phase against *M. tuberculosis* $H_{37}Rv$ challenge in mouse (CM, cell membrane; APP, aqueous phase proteins; DPP, detergent phase proteins; remaining details are the same as given in Table 1)

Groups	Viable bacilli count (CFU in Log_{10})		Survival (MST on day 30)
	Lungs	Spleen	
Control	11.611±0.045	8.283±0.160	11.89±1.92
CM	7.386±0.257 (1.57↓)	6.422±0.035 (1.28↓)	24.79±0.03 (2.08↑)
APP	6.252±0.357 (1.85↓)	5.034±0.626 (1.64↓)	26.56±1.45 (2.23↑)
DPP	7.509±0.066 (1.54↓)	6.641±0.055 (1.24↓)	24.34±1.35 (2.04↑)

in the two groups immunized with APP and DPP were compared with those immunized with whole membrane fraction or the unimmunized controls (Table 3). MST was significantly higher and CFUs were significantly lower in the two groups compared to the unimmunized group. In a comparison between whole membrane (CM) and its subfractions, survival rate of animals was highest in the APP group followed by CM and DPP groups. Lowering in CFUs in the lung and spleen of APP immunized mice was found statistically significant compared to the whole membrane fraction (Table 4). Similarly, CFUs in the two organs and MST of animals immunized with DPP were, respectively, higher and lower than the corresponding values in CM group these differences were however, not significant statistically. The reduction in CFU in lung and spleen in the APP group were statistically significant as compared to the DPP group Table 4.

4. Discussion

In the present study results of initial protection experiments revealed that antigens which provide resistance against *M. tuberculosis* infection in mice are localised in greater density in the membrane fraction of *M. habana*. Considering the technique used, the membrane fraction is least likely to be contaminated by cell wall and cytosol. On the other hand, there are fair chances of contamination of cell wall and cytosol preparations with the membrane antigens which may have influenced their protective efficacies.

These observations provided a lead for further characterization of antigens (proteins) of CM fraction of *M. habana*. Membrane was subjected to a gentle extraction using Triton X-114 [11,20] to separate proteins of peripheral and integral compartments. A major proportion of total membrane proteins (about 68%) constitute the peripheral compartment recovered in the aqueous phase. Proteins of the integral compartment which constitute 31% of the total membrane protein were identified as conjugates of lipopolysaccharides e.g. LAM, LM and PIMs. Dominant 38, 35 and 19 kD proteins of AP and 38, 27 and 12/10 kD proteins of DP may be of greater importance in view of vaccine development because proteins of corresponding loci have also been reported in *M. tuberculosis* [12,27–29]. The 38 kD protein has been isolated biochemically as well and reported as an immunodominant antigen of *M. tuberculosis* [28,29]. 38 and 35 kD protein have been reported to possess both T- and B-cell epitopes [27–29]. Moreover, reactivity (in ELISA, data not shown) with a battery of monoclonal antibodies against *M. tuberculosis* (provided by Dr. J. Ivanyi, London) revealed that antibodies TB78 and TB68 (against 65 and 16 KD antigens, respectively) were recognised by peripheral membrane antigens whereas TB23 (against 19 KD antigen) was recognised by integral membrane antigens of *M. habana*.

After essential confirmation and characterization of the membrane proteins, an attempt was made to find out the exact location of protective antigens within the membrane fraction. For this, protective efficacies of antigenic proteins of aqueous and detergent phases were evaluated against tuberculosis infection in mouse. To avoid any bias, equal protein doses of both phases (5 μg/mouse) were used for immunization. Results of protection experiments suggest that protective proteins are distributed in both compartments with a preference for the peripheral compartment. The protection was apparent by good physical condition, e.g. agility and body weight recorded weekly (data not shown), higher survival and lesser number of tubercle bacilli (in two vital organs, lung and spleen) after the virulent challenge in immunized mice. Lower protection offered by DPP may partly be explained by the presence of known immunosuppressive moieties, e.g. LAM and its products [6,7] in this fraction.

The observed phenomenon of protection by membrane proteins of *M. habana* against tuberculosis infection may be a result of enhancement of overall body immunity which takes care of the infection either by

Table 4
Statistical analysis for significance of variance between different immunised groups by "*t*" test (details are the same as given in Table 2)

	CFU		MST
	Lung	Spleen	
Membrane vs APP	$p < 0.05$	$p < 0.05$	NS
Membrane vs DPP	NS	NS	NS
APP vs DPP	$p < 0.05$	$p < 0.01$	NS

checking its onset and subsequent spread or by destroying the causative pathogen followed by faster clearance of the dead bacilli. This aspect is under study. Further, characterization of individual, protein molecule(s) of peripheral and integral compartments of *M. habana* membrane becomes mandatory.

Important finding of this study is the determination of subcellular location of protective antigens (proteins) of *M. habana* which play a major role in providing resistance against *M. tuberculosis* infection. Interestingly, separation pattern of peripheral and integral membrane proteins is same as that of *M. tuberculosis* $H_{37}Rv$ [12] which puts *M. habana* more close to *M. tuberculosis* and makes it a good choice as candidate vaccine for tuberculosis.

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